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Human Herpesvirus-6 Reactivation in a Longitudinal Study of Two HIV-1 Infected Patients

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After primary infection, human herpesvirus-6 (HHV-6) persists in latent form and can be reactivated in immunocompromised subjects. A longitudinal study of HHV-6 infection was carried out in two HIV-1 seropositive patients to provide in vivo evidence of HHV-6 reactivation. Concomitant with a significant rise of anti-HHV-6 IgG detected by IFA, a transient increase of HHV-6 viral load was shown in PBLs by PCR. During HHV-6 reactivation it was also identified either cell-free HHV-6 by PCR in plasma or IgM antibody titers. HHV-6 reactivation was followed by a temporary decrease in CD4+ count and by a progressive dramatic loss of CD4+ during the following 18 months. HHV-6 strain characterization by PCR demonstrated that first patient (MM) initially showed the B variant, followed by reactivation and persistence of the A variant, while in the second (SG) only the A variant was detected. The evidence of HHV-6 reactivation suggests its involvement in immunologic damage underlying the disease. ***J. Med. Virol. 51:259–264, 1997.***

KEY WORDS: HHV-6; HIV-1; viral reactivation; PCR; indirect immunofluorescence assay

INTRODUCTION

Human herpesvirus type 6 (HHV-6), first isolated from patients with both AIDS-associated and non-AIDS-associated lymphoproliferative diseases [Salahuddin et al., 1986], has been established as the etiologic agent of Exantem Subitum [Yamanishi et al., 1988] and a possible pathogenic role in other diseases is under investigation [Salahuddin et al., 1993].

HHV-6 establishes a latent presence in salivary glands and mononuclear blood cells after primary infection [Gopal et al., 1990; Jarret et al., 1990; Luppi et al., 1993]. Recently, it was observed that the HHV-6 full-length genome was integrated (at locus 17p13) in the PBMCs of non-AIDS-related lymphoma patients. This suggests a possible mechanism of viral latency in the host [Torelli et al., 1995]. Viral replication sometimes resumes in patients with one of several malignant and non-malignant diseases characterized by impaired immune functioning. HHV-6 reactivation is characterized by the presence of circulating infectious particles and a significant rise of antibody titers, especially in transplant patients [Asano et al., 1991; Okuno et al., 1990].

In immunodeficient patients the role of HHV-6 infection as a co-factor in the progression of HIV-1 infection is uncertain [Lusso and Gallo, 1995], because of its prevalent tropism for CD4+ cells [Takahashi et al., 1989] and its in vitro transactivating effect on HIV-1 replication [Ensoli et al., 1989]. Although HHV-6 has been isolated in some AIDS patients [Salahuddin et al., 1986; Downing et al., 1987; Agut et al., 1988] and active and disseminated HHV-6 infection has been observed in patients in the terminal stages of HIV-1 infection [Corbellino et al., 1993; Knox and Carrigan, 1994], to date no longitudinal studies document clearly HHV-6 reactivation and its consequences in the pathogenesis of AIDS.

The immunological, virological, and clinical outcomes were studied in two HIV-1 seropositive patients in whom there was reactivation of HHV-6 during follow-up.

MATERIALS AND METHODS

Patients

Patient MM, an HIV-1 seropositive woman, was asymptomatic at the time of reactivation of HHV-6. Her CD4+ count was 232 cells/mm^3 (CDC class A2) [Centers

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for Disease Control and Prevention, 1993]; she showed a progressive CD4+ cell depletion during follow-up, and she developed a wasting syndrome in the 16th month which lead to AIDS (CDC class C3 and 34 CD4+/mm^3, after 18 months).

Patient SG, an HIV-1 seropositive man, had developed AIDS, with *Pneumocystis Carinii* pneumonia, at the time of HHV-6 reactivation; his CD4+ count was 248 cells/mm^3 (CDC class C2). During follow-up he had a progressive CD4+ cell depletion and developed an AIDS-related esophageal candidiasis in the 13th month (CDC class C3 and 14 CD4+/mm^3 count, after 18 months).

Times 0 and −1 for patients MM and SG respectively, correspond to the start of anti-retroviral therapy. Previous plasma and PBL samples were not available.

Extraction of DNA from PBLs and plasma. Total cellular DNA was extracted from the peripheral leukocytes (PBLs) via proteinase K digestion and phenol-chloroform purification method at each time point of the follow-up study. DNA concentration was measured using spectrophotometry.

Five hundred microliters of plasma were centrifuged at 20,000 × *g* for 1 hr. The resulting pellet ws dissolved in PCR buffer using non-ionic detergents (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 200 μg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween 20) and digested using proteinase K (final concentration: 1 mg/ml) for 16 hr at 55°C. PCR was carried out after heat inactivation of proteinase K.

PCR Protocols

The amplificability of each DNA sample was tested by using B-actin gene primers [Kagi et al., 1994]. We amplified, via nested-PCR using P0-P4 as external primers and P1-P3 as internal primers, 1 μg of DNA extracted from PBLs and 10 μl of plasma crude extract to detect the ZVH14 fragment of HHV-6 genome [Josephs et al., 1986]. The first round amplification product measured 435 bp; the second measured 186 bp. The primer sequences and PCR protocol were as reported by Klotman et al. [1994].

Twenty microliters of the first round nested-PCR product were transferred onto Hybond N+ (Amersham International, UK) filters and hybridized with the P2 probe [Klotman et al. 1994], 3′ end labelled with ^{32}P. Hybridization and washing of filters was done as reported by Torelli et al. [1991]. The viral load was quantified in the PBLs by comparing the autoradiographic spots of the PCR products to those obtained by amplification of 10, 10^2, 10^3 copies of pZVH14 HHV-6 plasmid diluted in one μg of uninfected HSB-2 cell DNA.

All procedures were carried out in strict adherence to the recommendations of Kwok and Higuchi [1989] to avoid PCR contamination. PCR was carried out on negative controls, including uninfected HSB-2 cell DNA, PCR reaction without DNA, and DNA extraction reagents.

HHV-6 Strain Characterization

HHV-6 strains were identified and characterized by nested-PCR using C1-A1 as external and C2-A2 as internal primers [Secchiero et al., 1995], specific for a polymorphic viral region [Aubin et al., 1991]. The PCR protocol complied with that described by Secchiero et al. [1995]. The second round of nested-PCR products of 658 bp were digested with Hind III and Bgl II endonuclease enzymes. The restriction pattern was examined on 2% agarose gel stained with ethidium bromide.

Indirect Immunofluorescence Assay

In one test run patient sera were tested using indirect immunofluorescence assay (IFA) for anti-HHV-6 IgG and IgM after first removing the competing IgG. HHV-6 (GS strain) infected and uninfected HSB-2 cells [Ablashi et al., 1987], used respectively as positive and negative controls, were fixed on slides with acetone for 20 min at −20°C. Two-fold serial dilutions (from 1:10) of serum were applied to slides and incubated for 40 min at 37°C in a humidity chamber. The slides were washed twice with PBS (phosphate-buffered saline solution, pH 7.2), once with distilled water, and air dried. We added one drop of fluorescein-conjugated goat antibody anti-human IgG (Celbio, Camarillo, CA), diluted 1:50 with PBS (or anti-IgM diluted 1:20). We then incubated the slides for 40 min at 37°C. After two washes with PBS and one with distilled water, two operators (double blind) examined the slides with a fluorescence microscope.

The reciprocal of the highest positive serum dilutions was defined as the antibody titer. We considered an IgG titer equal or higher than 1:40 is positive and used 1:10 as the IgM positive titer cut-off.

RESULTS

The time of HHV-6 reactivation was defined as time 0 for patients MM and SG.

Serological Results

Both patients were seropositive for anti-HHV-6 IgG at the time of first analysis. Patient MM showed a significant increase of IgG titer, reaching a peak level at time 1 (1:640), corresponding to the higher positive titer (1:40) of IgM. We found detectable levels of anti-HHV-6 IgM 12 and 18 months after the acute reactivation and a constant IgG titer (Table I).

Patient SG had a rise of anti-HHV-6-IgG, reaching a peak level at time 1 (1:320) and showed detectable levels of IgM (1:10) at both time 0 and time 1. A positive titer of IgM was not reported until 18 months after acute reactivation (Table I).

PCR Results

All DNA samples showed efficient amplification for the b-actin gene (data not shown). Figure 1 (Panel B) shows autoradiographic detection of the first round of PCR products of 10, 10^2, and 10^3 ZVH14 plasmid copies. Our PCR method was able to detect the presence of as few as 10 copies of pZVH14 plasmid.

TABLE I. Serological Findings by Indirect Immunofluorescence Assay (IFA) in Sequential Plasma and PCR Results on PBLs and Plasma

Patient (HHV-6 variant)	Times	Titer of IgG (IFA)	Titer of IgM (IFA)	PCR PBLs[a]/ Plasma	CD4+ count
Patient MM					
B	0	1:40	1:10	++++/pos	232
A	1 (+32 days)	1:640	1:40	+++/pos	168
″	2 (+47)	1:320	1:10	++/neg	274
″	3 (+77)	1:160	<1:10	+/neg	260
″	6 months	1:160	<1:10	++/nd[b]	220
	12	1:80	1:20	nd/nd	90
″	18	1:80	1:10	+/nd	34
Patient SG					
	−1 (−27 days)	1:40	<1:10	−[c]/neg	174
A	0	1:80	1:10	+++/neg	248
″	1 (+14)	1:320	1:10	++/pos	206
″	2 (+30)	1:80	<1:10	+/neg	229
″	6 months	1:160	<1:10	++/nd	179
″	12	1:40	<1:10	++/nd	64
″	18	1:80	1:10	+/nd	14

*The semiquantitation of viral copy number is reported in the legend.
[a]Copy number/150,000 PBLs: ++++: $>10^3$; +++: 10^2–10^3; ++: 10–10^2; +: <10.
[b]nd: not determined.
[c]− = undetectable.

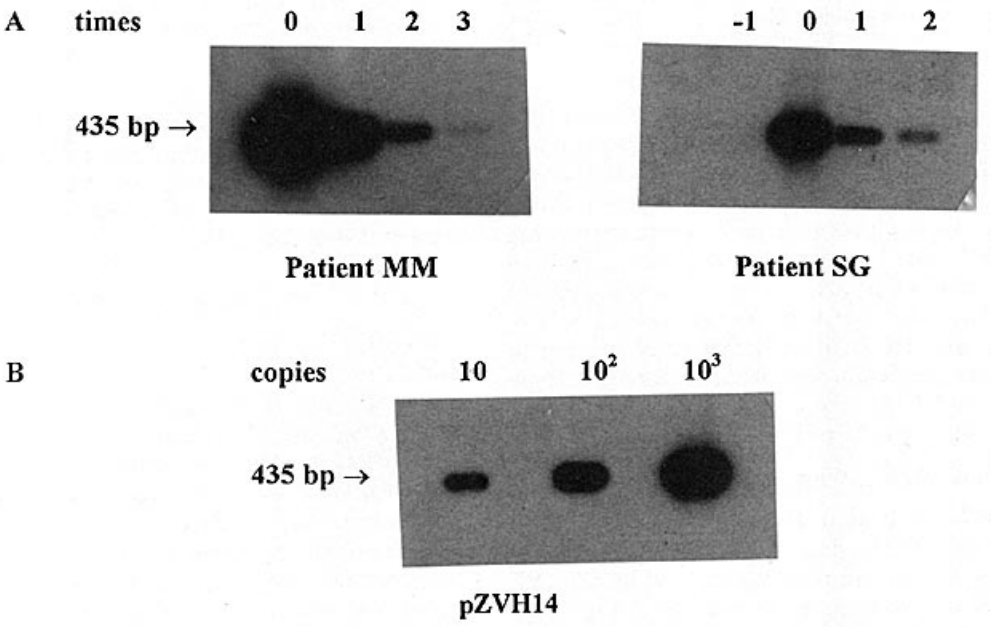

Fig. 1. **Panel A:** HHV-6 load progress in PBLs of patients MM and SG in periods of acute HHV-6 reactivation. Products of first round PCR (435 bp) were tansferred by Southern blot and hybridized with ^{32}P labelled P2. The times of follow-up are also reported in Table I; time 0 represents the point of HHV-6 reactivation. **Panel B:** Autoradiographic detection of first round nested-PCR products (435 bp) of diluition of pZVH14 plasmid copies (10, 10^2, 10^3), after hybridization with ^{32}P labelled P2.

Using etidium bromide staining on agarose gel after the second round of nested PCR, all samples that had scored positive by autoradiography were positive. All negative samples remained undetectable (data not shown). Blotting and autoradiography of the first round of PCR products were undertaken only when semiquantitative results were needed.

At time 0 patient MM was found positive for HHV-6 genomic sequences ($>10^3$ copy number/150,000 PBLs). At subsequent time points the viral load decreased, until, at time 3, less than 10 copy number/150,000 PBLs were found (Figure 1, Panel A). After time 3 the viral burden remained at low levels (6, 12, and 18 months after HHV-6 acute reactivation). HHV-6 DNA was found in plasma at times 0 and 1 (Table I).

At time −1 patient HHV-6 genomic sequences, were

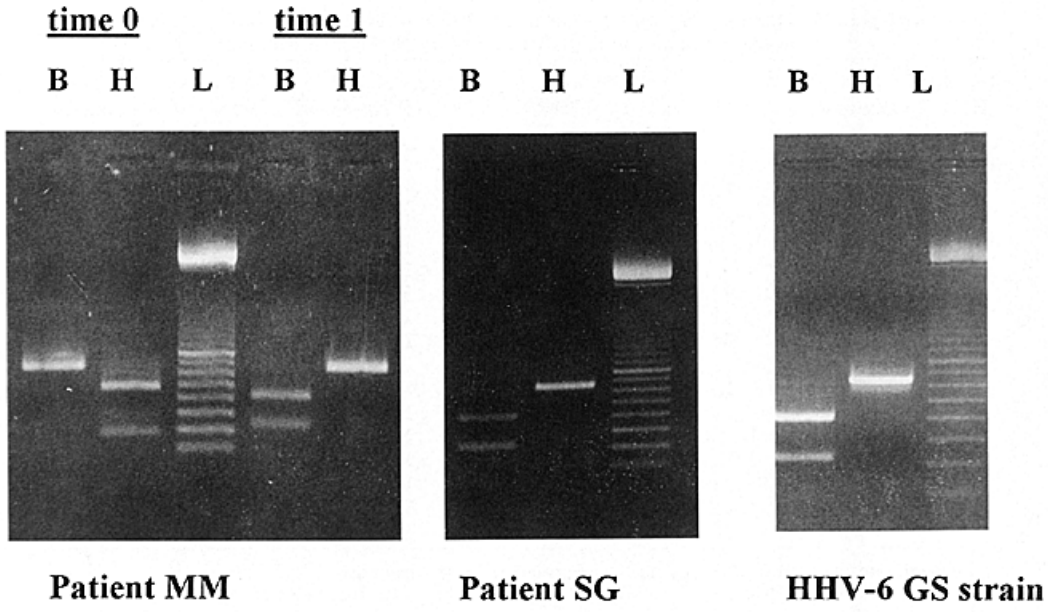

Fig. 2. HHV-6 strain characterization by digestion of 638 bp PCR products with Bgl II (B) and Hind III (H). Patient MM showed at time 0 a restriction pattern of B variant (Z29-like), while at time 1 showed the pattern of A variant (GS and U1102-like). At other times the patient also showed A variant (data not reported). Patient SG always showed the restriction pattern of A variant, as reported in the figure for the time 0. The restriction pattern of positive control (HSB-2/HHV-6, GS strain) was reported. L is the molecular weight ladder 100 bp (Pharmacia Biotech, Uppsala, Sweden).

not detected in patient SG but these were found at time 0 (10^2–10^3 copy number/150,000 PBLs). Subsequently, the viral load decreased, until, at time 2, less than 10 copy number/150,000 PBLs were found (Figure 1, Panel A). After the acute phase of HHV-6 reactivation the viral burden remained at low levels. At time 1, PCR on plasma revealed HHV-6 particles, while viral sequences were not detected at other time points (Table I).

Time points with the highest HHV-6 copy number in PBLs preceded significant rises of IgG titers. Furthermore, presence of viral sequences in the plasma was associated to specific anti-HHV-6 IgM.

HHV-6 Strain Characterization

In patient MM, at time 0, Hind III digestion of 658 bp fragment revealed the presence of two bands, while Bgl II digestion showed an undigested product (Fig. 2). However, at all the other times tested, Bgl II digestion of 658 bp fragment revealed two bands in each patient, while Hind III digestion showed undigested products. At time 0, patient MM showed an initial presence of HHV-6 genome of the B variant (Z29-like), while at subsequent time points only the presence of the A variant HHV-6 genome (GS and U1102-like strains) was detected. Patient SG showed the presence of A variant HHV-6 genome during all times following the HHV-6 reactivation.

CD4+ Cell Count and Clinical Conditions

Table I shows the CD4+ cell counts in patients MM and SG during the 18 months of follow-up. A slight decrease in the CD4+ count was observed immediately after HHV-6 reactivation, while no relevant clinical symptoms were showed during the acute phase. Within 18 months, the CD4+ count in both patients who developed AIDS-related diseases (wasting syndrome in patient MM and esophageal candidiasis in patient SG) decreased dramatically.

DISCUSSION

Like all other human herpesviruses, HHV-6 can remain latent in host cells after primary infection and may reactivate if the patient becomes immunosuppressed. Recently, Carrigan [1995] proposed that the reactivation of a latent virus can be documented by successful virus isolation, positive immunohistochemical staining, positive serum PCR, and strongly positive PCR for viral sequences in the infected cells. Using these methods, the reactivation of HHV-6 infection can be observed in two clinical subgroups, organ transplant recipients and AIDS patients.

Secchiero et al. [1995] introduced for the first time a semi-quantitative PCR method which allows the detection of HHV-6 sequences in the plasma of infected patients, providing a unique marker of active infection. They carried out a longitudinal study in three bone marrow transplantation patients, documenting the reactivation and monitoring the progression of HHV-6 infection in the setting of organ transplant in vivo.

The ability of this herpesvirus to enhance HIV-1 infection in vitro by various immunological and molecular mechanisms suggests a co-factorial role for HHV-6 in AIDS progression; however, the biological effects of HHV-6 infection in the clinical evolution of HIV-1 infection is still unclear [Lusso and Gallo, 1995]. Therefore,

a longitudinal study was undertaken in two HIV-1 infected patients to test for immunological and molecular markers of active HHV-6 infection.

Reactivation (or exogenous reinfection) of HHV-6 was observed in two HIV-1 seropositive patients, documented by molecular data (high viral burden in PBLs and presence of HHV-6 sequences in the plasma) and serological results (significant increase of IgG titers and detection of IgM titers).

The strong positivity of HHV-6 genome found by PCR revealed an active viral replication in PBLs, with presumably intracellular production of mature virions. After the time points with maximum viral burden anti-HHV-6 IgG titers increased significantly. Furthermore, during acute phase of HHV-6 reactivation, specific IgM antibodies and viral sequences were detected in plasma (Table I).

This report represents the first direct observation of HHV-6 reactivation of HIV-1 seropositive patients using a combination of serological and PCR assays. It is known that the determination of anti-HHV-6 antibody levels by ELISA an IFA methods is not sufficient *per se* to identify and HHV-6 carrier, since some antibody-negative subjects may harbor in their peripheral blood HHV-6 genomes that are detectable both by PCR and Southern blot [Luppi et al., 1993; Luppi et al., 1995]. Given the differences in PCR conditions [Luppi et al., 1995], useful information can be obtained only by appropriate use of this technique: evaluating differences in viral burden in a longitudinal study of a same subject.

Furthermore, after acute HHV-6 reactivation, we found that the HHV-6 copy number gradually decreased, along with the decline of the CD4+ count (6, 12, and 18 months). Fairfax et al. [1994] reported similar results. They reported, in HIV-1 seropositive patients, a HHV-6 copy number higher in patients with CD4+ cells $>400/mm^3$ than in patients with CD4+ cells $< 400/mm^3$. This could be because the loss of CD4+ cells, the main cellular target of HHV-6 replication, influences the detection of the viral burden in PBLs, but this does not exclude the possibility that HHV-6 could reactivate itself.

Data on HHV-6 in HIV-1 seropositive patients show a disseminated presence of HHV-6 infection in patients with terminal AIDS [Corbellino et al., 1993], even though an inverse correlation was found previously between anti-HHV-6 IgG titer and progression of HIV-1 infection [Spira et al., 1990]. This inverse correlation probably results from the failure of the immune system in the late stages of AIDS. If so, when HHV-6 replication occurs the humoral response would be limited, so that viral particles would disseminate throughout all tissues, infecting them productively [Knox and Carrigan, 1994]. Our results showed an initial HHV-6 reactivation, characterized by an increase of antibody titers, but with the progression of HIV-1 infection and the depletion of CD4+ cells, during 1 year of follow-up, we detected a normal level of IgG titers despite the fact that HHV-6 was still active, as shown by the presence of detectable IgM titers.

PCR strain analysis showed that the A variant of the HHV-6 genome in patients was associated with HIV-1 infection and the progression to AIDS. Initially, patient MM showed an acute reactivation of the B variant, but the A variant appeared after 1 month and persisted. Differences in viral burden of HHV-6 variants could explain this strange finding. In fact, if one variant is more prevalent than the other then only the most prevalent variant was revealed by PCR when using the same set of primers to detect both HHV-6 variants. Therefore, either a simultaneous or a sequential reactivation of both variants could have occurred in patient MM. Only the A variant was detectable and persisted during follow-up, suggesting it is associated with immunodeficiency progression as reported by Knox et al. [1995] in one pediatric case of HHV-6 infection. These observations lend support to in vitro findings reported by others, who observed an enhanced cell lysis in HHV-6, GS strain (A variant), and HIV-1 coinfected cells [Lusso et al., 1989] On the other hand, Secchiero et al. [1995] also described the prevalence of A variant during the active phase of HHV-6 infection in immunosuppressed patients.

In our patients, the acute phase of HHV-6 reactivation was asymptomatic, while a temporary decrease of CD4+ cell count was detectable later on. In the 12 months following HHV-6 acute reactivation, the fall of the CD4+ cell count and the rapid clinical disease progression were significant. Blazquez et al. [1995] reported similar results. They described one HIV-1 infected patient who had an inversion of CD4/CD8 ratio and a loss of CD4+ cells, coinciding with HHV-6 infection. Nevertheless, further studies are needed to determine if the initial decrease of CD4+ count is closely related to HHV-6 reactivation.

Even if it is difficult to establish how much the rapid progression of HIV-1 infection depends on HHV-6 reactivation, considering that HHV-6 is active in lymph nodes of HIV-1 infected patients [Knox and Carrigan, 1994], it is possible that HHV-6 infection can contribute to the destruction of lymph node architecture caused mainly by HIV-1 [Pantaleo et al., 1993]. Considering recent in vitro findings [Flammand et al., 1995] showing the immunosuppressive effect of HHV-6 on T-cell function (through suppression of IL-2 synthesis), it is possible that HHV-6 reactivation could be a strong signal of worsening immunological status.

The study of two HIV-1 positive patients suggests that the identifying markers of HHV-6 reactivation will prove to be a valuable tool in managing AIDS patients, especially for assessing clinical progression.

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